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# Adrenomedullin decreases extracellular signal-regulated kinase activity through an increase in protein phosphatase-2A activity in mesangial cells

Narayanan Parameswaran [a], Ponnal Nambi [b], Carolyn S. Hall [a], David P. Brooks [b], William S. Spielman [a,*]

[a] *Department of Physiology, Michigan State University, East Lansing, MI 48824, USA*

[b] *Department of Renal Pharmacology, SmithKline Beecham Pharmaceuticals, UW 2521, 709 Swedeland road, PO Box 1539, King of Prussia, PA 19406-0939, USA*

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**Abstract**

Adrenomedullin is a recently identified peptide hormone that has receptors in a number of different systems including renal mesangial cells. We reported recently that adrenomedullin can cause a decrease in extracellular signal-regulated kinase (ERK) activity and increase jun amino-terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK) acitivities in rat mesangial cells. Associated with these responses we also reported that adrenomedullin can decrease proliferation and increase apoptosis in mesangial cells. The major aim of the present study was to examine the mechanism of decrease in ERK activity by adrenomedullin and to identify the role of protein phosphatase 2A (PP2A) in the decrease in ERK activity, using okadaic acid [9,10-Deepithio-9,10-didehydroacanthifolicin], a selective inhibitor of PP2A at low nanomolar concentrations. The adrenomedullin-induced decrease in [$^3$H]-thymidine incorporation and increase in apoptosis were reversed by okadaic acid at the concentration that selectively inhibits PP2A. Okadaic acid completely reversed the ERK inhibition caused by adrenomedullin, suggesting that PP2A may be involved in the adrenomedullin-mediated changes in proliferation, apoptosis and ERK activity. PP2A activity in mesangial cells was increased over time following exposure to adrenomedullin. The tyrosine phosphorylation of ERK did not change significantly following adrenomedullin treatment although the ERK activity was decreased significantly. This suggests that the decrease in ERK activity is not mediated through a decrease in MEK (a dual phosphorylating kinase upstream of ERK) or by an increase in MKP-1/2 (a dual specificity phosphatase) activities. Thus we conclude that the mechanism of adrenomedullin-induced decrease in ERK activity in rat mesangial cells is at least in part mediated by an increase in PP2A activity.


*Keywords:* Okadaic acid; Adrenomedullin; ERK (extracellular signal-related kinase); JNK (jun amino-terminal kinase); P38 MAPK (P38 mitogen-activated protein kinase)

## 1. Introduction

Adrenomedullin is a 52 amino acid peptide derived from the precursor proadrenomedullin. It is a potent vasodilator and a natriuretic factor. It belongs to the Calcitonin Gene-Related Peptide (CGRP) superfamily (Kitamura et al., 1993; Sakata et al., 1993). In rat mesangial cells, adrenomedullin increases cAMP and causes a protein kinase A-dependent decrease in proliferation and an increased apoptosis (Parameswaran et al., 1999).

Associated with the changes in proliferation and apoptosis, adrenomedullin decreases extracellular signal-regulated kinase (ERK) activity and increases jun amino-terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK) activities in mesangial cells (Parameswaran et al., 1999). Because the activities of the mitogen-activated protein kinases (MAPKs) are regulated both by threonine and tyrosine phosphorylation, dephosphorylation of these sites could be a potential mechanism for down-regulating the activities of MAPKs (Waskiewicz and Cooper, 1995). Moreover, because the MAPKs are stimulated through a kinase cascade, which by themselves are regulated by phosphorylation/dephosphorylation mechanisms, phosphatases play a critical role in MAPK signaling (Mumby

* Corresponding author. Tel.: +1-517-355-6475 ext. 1210; fax: +1-517-355-5125.

*E-mail address:* spielman@psl.msu.edu (W.S. Spielman).

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and Walter, 1993; Wera and Hemmings, 1995). For example, protein phosphatase-2A (PP2A) is a serine/threonine phosphatase that dephosphorylates and decreases ERK activity. MAPK phosphatases 1 and 2 (MKP) are dual specificity phosphatases that can also dephosphorylate MAPKs. There are also other serine/threonine and tyrosine-phosphatases that have been shown to play a critical role in MAPK signaling (Hunter, 1995).

The major aim of the present study is to use a phosphatase inhibitor, specific for serine/threonine phosphatase, namely okadaic acid [9,10-Deepithio-9,10-didehydroacanthifolicin], to examine its effects on adrenomedullin-mediated responses. We demonstrate here for the first time that adrenomedullin increases PP2A activity in rat mesangial cells and that this could be a potential mechanism of adrenomedullin-mediated decrease in ERK activity and proliferation.

## 2. Materials and methods

### 2.1. Materials

Adrenomedullin was purchased from Phoenix Pharmaceuticals (Belmont, CA), myelin basic protein was purchased from Sigma (St. Louis). Polyclonal anti-ERK2, anti-P38 MAPK and anti-JNK1 antibodies were purchased from Santa Cruz laboratories (Santa Cruz, CA). Glutathione *S*-transferase conjugated-cJUN (GST-cJUN) was purchased from Alexis Biochemicals (San Deigo, CA). RPMI-1640, fetal bovine serum, penicillin and streptomycin were from Gibco (Grand Island, NY). All other reagents were of high quality available.

### 2.2. Cell culture

Rat mesangial cells were obtained from the glomeruli of kidney cortex isolated from Sprague Dawley rats as described before (Albrightson et al., 1992), and were grown in RPMI-1640 with 15% fetal bovine serum. Passages between 15 and 30 were used for the experiments.

### 2.3. [$^3$H]-thymidine incorporation

Cells were plated in 24 well plates (30 000 cells/well) and grown for 2 days after which they were serum starved for 48 h. Then, they were treated with the compounds for a period of 16 h and pulsed with [$^3$H]-thymidine for 4 h. The radioactivity was counted in Beckman LS counter, after washing the cells and stopping the reaction with 5% trichloro acetic acid and solubilizing the cells in 0.5 ml of 0.25 N sodium hydroxide. Each experiment was done in quadruplicates and was repeated at least three times.

### 2.4. Kinase assays

Cells were plated in p100 plates and were serum starved overnight on reaching confluency. The agonist solutions were prepared in the growth media without serum. Cells were treated with the agonists for 30 min. The cell lysates were prepared as described (Bogoyevitch et al., 1995; Li et al., 1995). In the meantime, specific antibodies (10 μg/reaction) were incubated with protein A agarose (Gibco) for 30 min at room temperature. After normalizing for protein concentration, the cell lysates were incubated with the specific antibody agarose conjugate for 2 h at 4°C with constant shaking. The kinase assays were done after washing the immunoprecipitate three times with HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) buffer and two times with kinase buffer (50 mM Tris–HCl, 100 mM NaCl, 10 mM $MnCl_2$ and 0.1 mM sodium ortho vanadate). The functional assay was done in the presence of 50 μM ATP, 5 μCi $^{32}$P-ATP, 10 μg of specific substrate (MBP for ERK2 and P38-MAPK, and GST-cJUN for JNK1), and the immunoprecipitate. The reactions were performed at 30°C for 15 min and then stopped with sodium dodecyl sulfate buffer. The samples were then electrophoresed on 12% polyacrylamide gel and the gels were dried and subjected to autoradiography or phosphoimager plates. The intensity of the bands in the autoradiogram was visualized using an ARCUS high-resolution optical scanner and quantitated using NIH image software or quantitated using imagequant program (for the gels exposed to phosphoimager plates). Results are expressed as percent change from the basal of the relative densitometric units or phophoimager units.

### 2.5. Western blots

Western blot analysis was done as described before (Guo et al., 1998). Briefly, equal concentration of protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then blocked with 5% non-fat dry milk in tris-buffered saline containing 0.05% Tween-20 and incubated with primary antibodies followed by horse radish peroxidase-conjugated secondary antibodies according to manufacturer's instructions. The blots were then visualized by an enhanced chemiluminescence (ECL) kit obtained from Pierce. For tyrosine phosphorylation of ERK, ERK was immunoprecipitated with ERK2 antibody, run on a gel, transferred and then probed for tyrosine phosphorylation with anti-phospho tyrosine antibody. The blot was then stripped and reprobed for ERK using anti-ERK antibody. The phosphotyrosine bands were then normalized with the ERK expression.

### 2.6. ELISA for apoptosis

The ELISA kit was obtained from Boehringer Mannheim (Indianapolis, IN), which specifically detects

the cytoplasmic nucleosomal DNA. For that, cells were plated in 48 well plates and after 24 h were serum starved for another 24 h. Different agonists (prepared in the media) were added to the cells and the cells were incubated for another 18 h. The cells were lysed with the lysis buffer and centrifuged to separate cytoplasmic and nuclear fractions. The cytoplasmic fraction was then tested for DNA still attached to nucleosomes using the ELISA protocol from Boehringer Mannheim, Indianapolis, IN. The assay was done in triplicates or quadruplicates and repeated at least 3–5 ×.

### 2.7. PP2A activity

The kit for PP2A activity was obtained from Upstate Biotechnology (Lake Placid, NY). PP2A activity assay was done as described before (Chajry et al., 1996). Briefly, the cell lysates were prepared without any phosphatase inhibitors, after treatment with adrenomedullin for the indicated time points. Equal amounts of protein lysates were then immunoprecipitated in triplicates. The immunoprecipitation protocol was same as that of the kinase assay. After that, the phosphatase activity was measured in the presence of a specific Ser/Thr-phosphopeptide and the phosphate released was determined colorimetrically using malachite green at 405 nm in a plate reader. The experiment was done in triplicates and repeated at least three times.

## 3. Results

### 3.1. [$^3$H]-thymidine incorporation

Adrenomedullin caused a significant decrease in [$^3$H]-thymidine incorporation in rat mesangial cells. Okadaic acid at 1.25 nM (a concentration that selectively inhibits PP2A) completely reversed the effect of adrenomedullin on proliferation (Fig. 1A). Okadaic acid treatment alone at these low concentrations caused a slight increase in proliferation which was not statistically significant (Fig. 1A). A

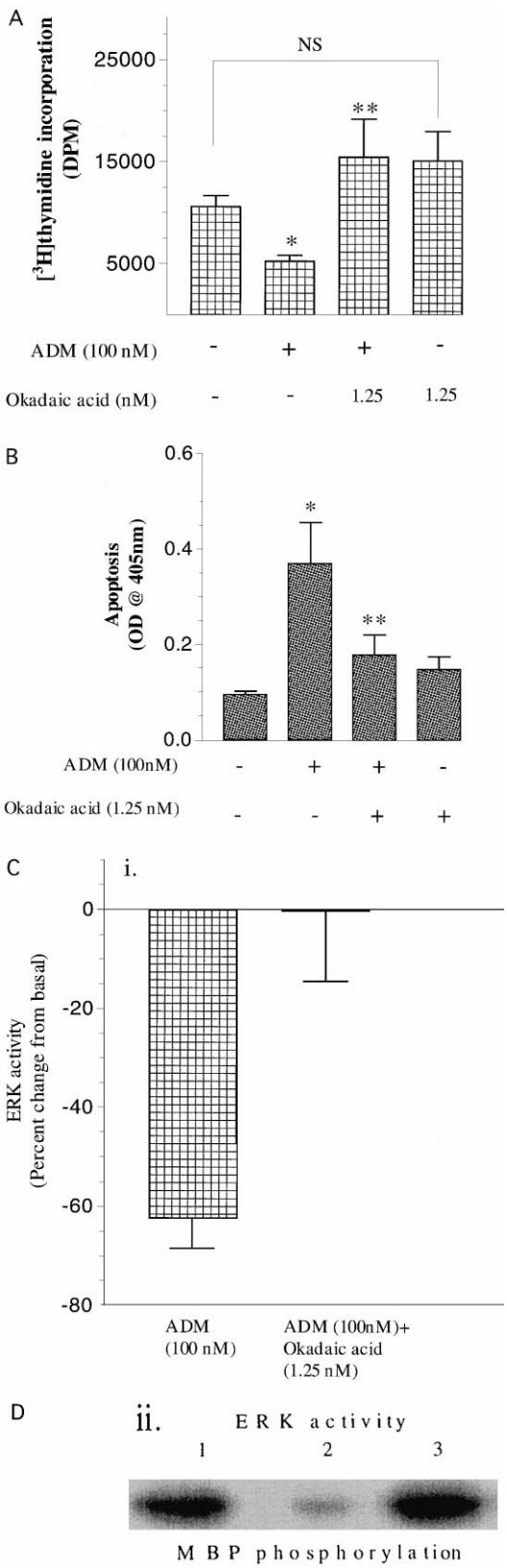


Fig. 1. (A) Effect of adrenomedullin (ADM) and okadaic acid (Ser/Thr phosphatase inhibitor) on [$^3$H]thymidine incorporation in rat mesangial cells. Experiment was done as described in the methods section. Okadaic acid was added 30 min before ADM treatment. ADM caused a significant decrease in thymidine incorporation ($n = 3$). $^*P < 0.01$ compared to basal, $^{**}P < 0.05$ compared to ADM. NS = not significant. (B) Effect of ADM and okadaic acid on cytoplasmic nucleosome-associated DNA fragmentation (an index of apoptosis) in rat mesangial cells. Experiment was done as described in the Methods section. Okadaic acid was added 30 min before ADM treatment. ADM caused a significant increase in DNA fragmentation and okadaic acid significantly inhibited ADM-stimulated apoptosis. The experiments were done in triplicates and repeated four times. A representative experiment is shown. $^*P < 0.01$ compared to basal; $^{**}P < 0.01$ compared to ADM. (C) (i) Effect of ADM and okadaic acid on ERK2 activity in rat mesangial cells ($n = 4$). Experiment was done as described in the methods section. Okadaic acid (OA) was added 30 min before ADM treatment. OA by itself did not affect ERK2 activity. $^*P < 0.01$ compared to ADM. (ii) A representative autoradiogram showing the effect of ADM and OA on ERK2 activity in rat mesangial cells. Lanes: 1 = Basal, 2 = ADM, 3 = ADM + OA(1.25 nM).

small increase in proliferation by okadaic acid itself is not surprising because there is basal PP2A activity and inhibiting that could lead to an increase in proliferation.

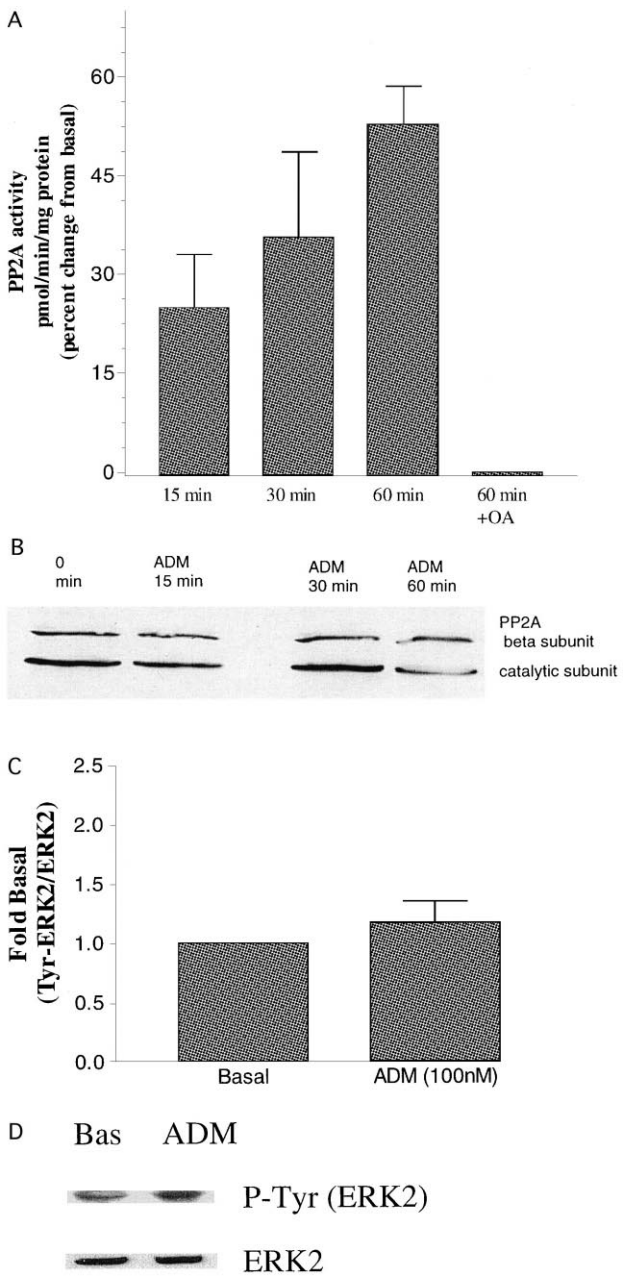


Fig. 2. (A) Effect of ADM on immunocomplex-associated PP2A activity in rat mesangial cells. ADM caused a time-dependent increase in specific PP2A activity. OA treatment (1.25 nM) after immunoprecipitation and before the phosphatase assay completely inhibited PP2A activity. (B) Immunoblots (Western) showing the effect of ADM on PP2A at the protein level. ADM increased PP2A activity without increasing the protein expression levels. (C) Effect of ADM on tyrosine phosphorylation of immunoprecipitated ERK2 in rat mesangial cells. Although ADM decreased ERK2 activity significantly, tyrosine phosphorylation of ERK2 did not change ($n = 4$). (D) Representative immunoblot (Western) showing the effect of ADM on tyrosine phosphorylation of immunoprecipitated ERK2 in rat mesangial cells. Top panel is the blot probed with P-Tyr antibody. The same blot was stripped and re-probed for ERK2 to normalize the tyrosine phosphorylation to ERK2 expression.

### *3.2. Cytoplasmic nucleosome-associated DNA fragmentation*

Adrenomedullin also caused a significant increase in DNA fragmentation (an index of apoptosis) and okadaic acid at 1.25 nM inhibited adrenomedullin-induced apoptosis in mesangial cells (Fig. 1B). By itself, at this low concentration okadaic acid did not affect rat mesangial cell apoptosis (Fig. 1B).

### *3.3. MAPK activities*

While okadaic acid did not have any effect on adrenomedullin-induced JNK1 and P38 MAPK acitivities (data not shown), it affected ERK2 activity (Fig. 1C). It completely abolished adrenomedullin-mediated inhibition of ERK activity. None of the kinase activities were affected by okadaic acid pretreatment alone (data not shown).

### *3.4. PP2A activity*

Because okadaic acid has been shown to be a specific inhibitor of PP2A at low nanomolar concentrations (the same concentration that we have used in our studies), we hypothesized that at least part of the effect at this low concentration on ERK2 activity could be explained through an increase in PP2A activity. In fact, using specific antibodies for PP2A, we found that adrenomedullin does induce a time-dependent increase in PP2A activity (Fig. 2A), and that the PP2A activity can be completely inhibited by 1.25 nM okadaic acid (Fig. 2A). The increase in activity was without an increase in PP2A expression (Fig. 2B).

### *3.5. Tyrosine phosphorylation of ERK*

To examine the role of the other components of the ERK cascade (namely Raf, MEK, and MKP-1/2), and PP2A on adrenomedullin-mediated decrease in ERK activity, we hypothesized that if the decrease in ERK activity was primarily dependent on PP2A, then the tyrosine phosphorylation of ERK wouldn't change after adrenomedullin treatment. On the other hand, if the decrease is mediated primarily through an increase in MKP1/2 activity or a decrease in Raf and MEK activity then the tyrosine phosphorylation would also decrease for ERK because MKP is a dual specificity phosphatase and MEK is a dual specificity kinase. But as shown in Fig. 2C, although ERK activity is decreased by adrenomedullin, tyrosine phosphorylation of ERK did not change suggesting a role for PP2A in the decrease in ERK activity.

## 4. Discussion

The MAPK cascade generically includes a small GTP binding protein such as ras, activating a MAPK kinase kinase (MAPKKK), which subsequently activates a MAPK

Kinase and then a MAPK. Until now three parallel pathways, namely the ERK, JNK and P38 MAPK pathways have been well described. cRaf is a MAPKKK that activates MAPKK which then activates ERK (a MAPK) (Robinson and Cobb, 1997). Since the activation and deactivation of the entire MAPK cascade involves phosphorylation and dephosphorylation at various steps, it is obvious that phophatases also play a critical role in this cascade (Hunter, 1995). Haneda et al. (1996) showed that adrenomedullin can decrease MEK activity in mesangial cells. We reported that adrenomedullin can decrease ERK2 activity and increase JNK and P38 MAPK activities. The decrease in ERK and increase in JNK and P38 MAPK activities were associated with a decrease in proliferation and an increase in apoptosis in rat mesangial cells (Parameswaran et al., 1999). The present investigation was undertaken to understand: (a) the mechanisms underlying the changes in the ERK activity and the associated changes in proliferation and apoptosis, and (b) the involvement of PP2A in adrenomedullin-mediated responses. To inhibit PP2A, we have used okadaic acid which is a fairly selective inhibitor of protein phosphatase 2A [PP2A] ($IC_{50}$ 0.1 nM) and protein phosphatase 1 [PP1] ($IC_{50}$ 10 nM) (Hunter, 1995; Wera and Hemmings, 1995). Okadaic acid also inhibits PP2B at very high concentrations (Hunter, 1995). Okadaic acid has been shown to have tumor promoting as well as apoptotic effect depending on the system (Amaral et al, 1993). At concentrations as low as 1.25 nM (that selectively inhibits PP2A), okadaic acid reversed the effects of adrenomedullin on ERK2 activity, [$^3$H]-thymidine incorporation (proliferation) and cytoplasmic nucleosome-associated DNA fragmentation (apoptosis) in rat mesangial cells. Furthermore adrenomedullin caused a time-dependent increase in PP2A activity in mesangial cells, and this activity was also completely inhibited by 1.25 nM okadaic acid. This indicates that the likely mechanism of the effect of okadaic acid at this low concentration is through inhibition of PP2A (a serine/threonine specific phosphatase). In further support of this hypothesis, we provide an indirect evidence that although adrenomedullin decreases ERK activity it does not change the tyrosine phosphorylation of ERK. This suggests that neither a decrease in MEK (a dual phosphorylating kinase) nor an increase in MKP activity (a dual specificity phosphatase) or any other tyrosine specific phosphatase are primarily responsible for the decrease in ERK activity. If they were, then the decrease in activity would have correlated with the decrease in tyrosine phosphorylation. Also, it is unlikely that at this low concentration, PP1 is inhibited ($IC_{50}$ for PP1 is 10 nM). Hence, adrenomedullin-stimulated PP2A might be primarily responsible for a decrease in ERK2 activity, although we are not precluding other pathways including MKP-1, that may also decrease the ERK activity. We think this could also be a direct mechanism because of the evidence from the literature that PP2A can directly dephosphorylate ERK and decrease its activity (Hunter, 1995; Waskiewicz and Cooper, 1995). The fact that at this low concentration, okadaic acid did not affect other kinase activities, namely, the JNK and P38, indicates that adrenomedullin-stimulated PP2A activity is not involved in the signaling pathways leading to JNK and P38 activation.

Our earlier findings using a P38 MAPK inhibitor (SB203580) {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole} indicated that both proliferation and apoptosis can be mediated through P38 MAPK [Parameswaran et al., 1999]. Based on our results with okadaic acid and SB203580 {[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole}, it appears that both ERK and P38 MAPK may regulate adrenomedullin-mediated changes in proliferation and apoptosis. Inhibition of ERK and/or activation of P38 MAPK may cause a decrease in proliferation and increase in apoptosis of mesangial cells.

## 5. Conclusion

Adrenomedullin decreases ERK activity and proliferation, while it increases apoptosis in rat mesangial cells. Associated with this it also increases PP2A activity in a time-dependent manner. Inhibition of this increase in PP2A activity by okadaic acid reverses the effect of adrenomedullin on ERK activity, proliferation and apoptosis. Furthermore, adrenomedullin-mediated decrease in ERK activity is not accompanied by a decrease in tyrosine phosphorylation. These results suggest that at least part of the mechanism of adrenomedullin-mediated decrease in ERK activity is through an increase in PP2A activity and that this could be one of the potential mechanisms controlling mesangial cell proliferation and apoptosis.

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